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# Mechanism of Interferon Action: RNA-Binding Activity of Full-Length and R-Domain Forms of the RNA-Dependent Protein Kinase PKR — Determination of $K_D$ Values for $VA_I$ and TAR RNAs

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The RNA-binding activity of the interferon-inducible, RNA-dependent protein kinase PKR, expressed from the human PKR cDNA, was quantitated using a gel mobility-shift assay. The N-terminal R-domain truncation Wt(1–243) and the full-length catalytic mutant K296R(1–551) were analyzed for their abilities to bind adenovirus $VA_I$ RNA, human immunodeficiency virus TAR RNA, and the synthetic homopolymer pI:pC RNA. The N-terminal 243 amino acid residue form of PKR [Wt(1–243)] bound $VA_I$ RNA with similar affinity as the 551 amino acid residue full-length catalytic mutant [K296R(1–551)]. The dissociation constant for $VA_I$ RNA was approximately $2 \times 10^{-9}$ *M* for both the K296R(1–551) and Wt(1–243) proteins. The K64E mutation significantly impaired the $VA_I$ RNA-binding activity as measured with the full-length double-point mutant PKR protein, K64E/K296R(1–551). Using a gel-shift competition assay, the dissociation constants of K296R(1–551) and Wt(1–243) for $VA_I$(1–160) RNA and pI:pC RNA were comparable. By contrast, the dissociation constants of K296R(1–551) and Wt(1–243) for TAR(1–82) RNA were both about $1 \times 10^{-7}$ *M*. These results suggest that the RNA-binding affinity of PKR is approximately 100-fold lower for TAR RNA than for either $VA_I$ RNA or pI:pC RNA and that the full-length and N-terminal R-domain forms of PKR bind RNA with similar affinity. 

## INTRODUCTION

The IFN-induced RNA-dependent protein kinase (PKR)[3] is dependent upon RNA for activation of kinase activity, a process which involves the autophosphorylation of PKR (Samuel, 1993). Following activation, PKR catalyzes the phosphorylation of protein synthesis initiation factor eIF-2 at serine 51 of the $\alpha$ subunit (Samuel, 1979, 1993; Galabru and Hovanessian, 1987; Hershey, 1989; Pathak *et al.*, 1988). The PKR-catalyzed phosphorylation of eIF-2$\alpha$ leads to an inhibition of protein synthesis, characterized by the selective inhibition of translation of certain mRNAs in transfected cells (Kaufman *et al.*, 1989; Samuel and Brody, 1990).

PKR plays a central role in regulating protein synthesis in virus-infected cells (Katze, 1992; Samuel, 1991). Several viruses encode RNAs that are bound by PKR and either activate or antagonize the autophosphorylation of PKR (Katze, 1992; McCormack *et al.*, 1992; Mathews, 1993; Samuel, 1992). Among the viral RNAs that affect PKR catalytic activity are adenovirus $VA_I$ RNA, Epstein–Barr virus EBER RNA, reovirus s1 mRNA, and human immunodeficiency virus (HIV) TAR RNA (Schneider *et al.*, 1985; Galabru *et al.*, 1989; Clarke *et al.*, 1991; Bischoff and Samuel, 1989; Edery *et al.*, 1989; SenGupta *et al.*, 1990). A common feature of these viral RNAs is their ability to form extensive secondary structure.

The adenovirus $VA_I$ RNA is one of the most highly characterized effectors of PKR activity; the $VA_I$ RNA facilitates adenovirus late protein synthesis and antagonizes the antiviral actions of IFN through the inhibition of PKR activation (Mathews and Shenk, 1991; Samuel, 1991; Mathews 1993). At late times after adenovirus infection, double-stranded RNAs capable of activating PKR are formed by RNA polymerase II-catalyzed synthesis of the opposing transcripts of the viral genome (Maran and Mathews, 1988). However, viral protein synthesis is maintained by the RNA polymerase III-catalyzed production of large amounts of $VA_I$ RNA (Kitajewski *et al.*, 1986). $VA_I$ RNA binds to PKR, preventing activation and thus the subsequent phosphorylation of eIF-2$\alpha$ (Katze *et al.*, 1987). An adenovirus deletion mutant that does not synthesize the $VA_I$ RNA is unable to efficiently replicate when compared to wild-type virus (Kitajewski *et al.*, 1986). However, the replication of $VA_I$-deficient adenovirus is rescued in host cells which express the S51A mutant eIF-2$\alpha$ that cannot be phosphorylated at serine-51 (Davies *et al.*, 1989; Pathak *et al.*, 1988). The structure–function relationship of $VA_I$ RNA and its ability to inhibit PKR kinase activation has been examined *in vitro* and *in vivo* (Mathews, 1993). Both the apical stem of $VA_I$ RNA and the central core

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[3] Abbreviations used: PKR, the interferon-inducible RNA-dependent eIF-2$\alpha$ protein kinase; IFN, interferon; dsRNA, double-stranded RNA; bp, base pair; nt, nucleotide.



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have been implicated in the $VA_I$-mediated inhibition of PKR activation (Furtado *et al.,* 1989; Ghadge *et al.,* 1991; Pe'ery *et al.,* 1993; Ma and Mathews, 1993).

Mutational analyses of PKR cDNA clones have localized the RNA-binding activity to the N-terminal region of the kinase (McCormack *et al.,* 1992; Patel and Sen, 1992; Feng *et al.,* 1992; Katze *et al.,* 1991). Adenovirus $VA_I$ RNA, HIV TAR RNA, and reovirus sI RNA, three natural RNAs which affect PKR-enzymic activity (Samuel, 1991), are all bound by TrpE–PKR fusion proteins which include the N-terminal 98 residues of PKR. By contrast, none of the viral RNAs are bound by TrpE–PKR fusion proteins that lack the N-terminal region of PKR (McCormack *et al.,* 1992). The region of PKR which displays RNA-binding activity includes a 20 amino acid core motif, designated $R_II$, which is conserved and often repeated in RNA-binding proteins from both prokaryotic and eukaryotic cells and their viruses (McCormack *et al.,* 1992; St. Johnston *et al.,* 1992). Substitution of R motif consensus residues abolishes RNA-binding activity (Chang and Jacobs, 1993; McCormack *et al.,* 1994).

As an extension of our studies on the structure and function of the PKR kinase, we have analyzed PKR mutants for their RNA-binding activity using a gel mobility-shift assay. The results of gel mobility-shift analyses confirm and extend in a quantitative manner results obtained using a Northwestern gel-blot assay. The N-terminal 243 amino acid residues are sufficient to mediate RNA-binding activity comparable to the full-length 551 residue PKR protein; the K64E mutation significantly impairs RNA-binding activity. The dissociation constants of K296R(1–551) and Wt(1–243) are comparable for both natural and synthetic RNAs; however, adenovirus $VA_I$ and synthetic pI:pC are bound about 100-fold more efficiently than is HIV TAR RNA.

## MATERIALS AND METHODS

### Preparation of PKR for gel mobility-shift analysis

Recombinant PKR protein for RNA gel mobility-shift analysis was prepared from transfected COS cells. The construction of the pJC119 expression vectors used herein for the production of the PKR proteins was described previously: Wt(1–551) and K296R(1–551) (Thomis and Samuel, 1992); Wt(1–243), K64E(1–551), and K64E/K296R(1–551) (McCormack *et al.,* 1994). COS cells grown as monolayer culture (150-mm dishes, Nuclon) with DMEM + 5% FBS were transfected by the DEAE–dextran/chloroquine phosphate method using 40 $\mu$g of DNA per 4 ml of serum-free DMEM (Luthman and Magnusson, 1983; Munemitsu and Samuel, 1988). Three culture dishes were routinely transfected with each pSV(PKR) construction; harvest was at 48 hr post-transfection. Recombinant PKR proteins were isolated from the ribosomal salt-wash fraction essentially as previously described (Samuel *et al.,* 1986). The 0.8 *M* ribosomal salt-wash fraction from transfected cells containing recombinant PKR protein was chromatographed on a DEAE-cellulose column, a step which removed endogenous nucleic acids and further purified the PKR from other proteins. Eluate fractions containing PKR were pooled, aliquoted, frozen in $LN_2$, and stored at −80°.

The relative concentrations of the partially purified recombinant PKR protein preparations were determined by Western immunoblot analysis using both monoclonal antibody 71/10 (kindly provided by Dr. Ara Hovanessian, Paris) and polyclonal PKR antisera (Thomis *et al.,* 1992). The PKR Western signals were compared to a PKR standard of known concentration, purified HIS-K296R(1–551) (Thomis and Samuel, 1993), in order to determine the molar concentration of the recombinant PKR proteins.

### Preparation of RNA

The transcription vectors pT7$VA_I$ and pT7TAR were used to prepare the adenovirus $VA_I$(1–160) RNA and the HIV TAR(1–82) RNA as previously described (McCormack *et al.,* 1992). For gel mobility-shift analysis, the RNAs were purified by fractionation on 6 *M* urea–10% polyacrylamide gels. Heterogenous pI:pC RNA obtained from the Antiviral Substances Program, NIH, was size-fractionated by 7 *M* urea–polyacrylamide gel electrophoresis.

### Gel mobility-shift analysis

Gel mobility-shift analyses were performed essentially as described (Gatignol *et al.,* 1993) with the following modifications. The final reaction mixture (10 $\mu$l) contained approximately 1 ng of recombinant PKR protein and varying concentrations of $^{32}$P-labeled $VA_I$ RNA probe (sp act $>1 \times 10^7$ cpm/pmol) in 15 m*M* Tris–HCl, pH 7.8, 70 m*M* NaCl, 10 m*M* KCl, 1 m*M* EDTA, 6% glycerol (v/v), 0.01% Triton X-100 (v/v), 25 ng ovalbumin, 2 units rRNasin (Promega), and 10 ng of Torula yeast RNA. Gel-shift reaction mixtures were incubated for 20 min at room temperature before loading onto a 10% native polyacrylamide gel (80:1 acrylamide–bisacrylamide) in 0.5× Tris–borate EDTA buffer. The gel was prerun at 4° for >1 hr at 200 V; 40 ml of a 2% glycerol solution was added to the wells prior to loading the samples. Electrophoresis was at 280 V for 5 hr or until the xylene cyanol marker was approximately 3 cm from the bottom of the gel. Gels were then dried and subjected to autoradiography. In gel-shift competition experiments, the competitor RNA was added at the same time as the $^{32}$P-labeled $VA_I$ RNA probe.

### Determination of dissociation constants ($K_D$)

Gel-shift analysis was utilized to determine the dissociation constants of the PKR proteins K296R(1–551) and Wt(1–243) for adenovirus $VA_I$ RNA. Dissociation constants were calculated by regression analysis of the relative amount of PKR–RNA complex formed in the pres-

ence of varying amounts of $^{32}$P-labeled $VA_I$ RNA (Reich *et al.*, 1992). Following autoradiography, the $VA_I$ RNA probe and the PKR–$VA_I$ complexes were excised from the dried gel using the autoradiogram as a template. The excised gel slices were measured for $^{32}$P Cerenkov counts using a Beckman LS 1801 system. Binding data was analyzed by the KinetAsyst program (IntelliKinetics, State College, PA).

The dissociation constants of K296R(1–551) and Wt(1–243) proteins for $VA_I$(1–160) RNA, TAR(1–82) RNA, and pI:pC RNA were also determined by competition analysis. This analysis was also performed with $^3$H-labeled $VA_I$ as the competitor to confirm the results obtained by the direct titration of $^{32}$P-labeled $VA_I$ RNA. The MINSQ software from Micromath Science Software was used to calculate the $K_I$ for both pI:pC RNA and TAR RNA, using $^{32}$P-labeled $VA_I$ RNA as the probe. The quadratic equation describing the coupled equilibria, shown below for PKR–$VA_I$ (EC) and PKR–competitor (EI), was recently described by Reich *et al.* (1992).

$$K_C = [E][C]/[EC]$$

$$K_I = [E][I]/[EI]$$

$$[I] = I_o - [EI]$$

$$[EI] = E_o - [EC] - [E] = E_o - [EC] - (K_c[EC]/[C])$$

$$[EC]^2\{K_c(1 + K_c/[C]\} + [EC]\{K_c(I_o - E_o + KI) + K_I[C]\} - K_I[C]E_o = 0.$$

By using the quadratic equation to solve for EC we are able to use this model to fit the experimental data of EC versus $I_o$, to obtain the value for $K_I$.

$$EC = \frac{-\{K_c(A_o - E_o + K_I) + K_I[C]\} \pm \{(K_c(A_o - E_o + K_I) + K_I[C])^2 - 4(K_c + K_c^2/[C])(-K_I[C]E_o)\}^{1/2}}{2(K_c + K_c^2/[C]}$$

## RESULTS

### Gel-shift analysis of recombinant PKR as a function of $VA_I$ RNA probe concentration

To determine the $K_D$ of the Wt(1–243) protein for the adenovirus $VA_I$ RNA, gel mobility-shift assays were performed using varying concentrations of $VA_I$ RNA ligand and a constant concentration of PKR protein. The concentration of recombinant PKR protein was determined by quantitative Western analysis, using both a monoclonal PKR antibody (Fig. 1) and polyclonal PKR antiserum (data not shown) with purified HIS-K296R(1–551) protein as an internal standard.

The Wt(1–243) protein bound $VA_I$ RNA as two specific complexes, designated complex I and complex II (Fig. 2A, lanes 2–12). The formation of complex I and II was dependent upon the concentration of the $VA_I$ RNA in the gel mobility-shift reaction. Both complexes I and II were present when the RNA was limiting (Fig. 2A, lanes 2–8), but when the $VA_I$ RNA was in excess, only complex I was formed (Fig. 2A, lanes 9–12). Complexes I and II were formed in comparable amounts at a $VA_I$ RNA concentration of $7.7 \times 10^{-10}$ *M;* the addition of PKR monoclonal antiserum supershifted both complex I and complex II (Fig. 2A, compare lanes 7 and 14). When an endogenous COS cell protein preparation isolated from cells, not transfected with PKR, was examined as a control, a minor protein species which bound $VA_I$ RNA, designated RSW for ribosome salt wash, was detected (Fig. 2B). However, the endogenous RSW complex was not supershifted by the PKR monoclonal antibody (Fig. 2B, lane 14). Prior studies revealed that endogenous expression of monkey PKR in COS cells was extremely low relative to vector-encoded PKR expression (Thomis and Samuel, 1992; McCormack *et al.*, 1994).

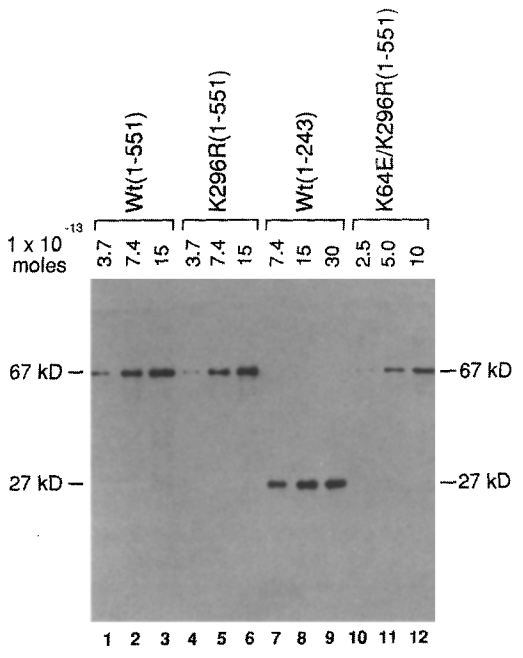


FIG. 1. Determination of PKR concentration. Recombinant PKR proteins isolated from transfected COS cells and fractionated by DEAE-cellulose chromatography were separated by $NaDodSO_4$–PAGE and subjected to Western analysis using monoclonal antisera and the ECL Western blotting procedure (Amersham) as described under Materials and Methods.

### Gel-shift analysis of recombinant K296R(1–551) and K64E/K296R(1–551) as a function of $VA_I$ RNA probe concentration

RNA gel mobility-shift assays were performed using a constant concentration of K296R(1–551) and varying concentrations of $VA_I$ RNA (Fig. 3A). The K296R(1–551) PKR protein formed both complexes I and II when the $VA_I$ RNA was limiting (Fig. 3A, lanes 2–6), but only complex I

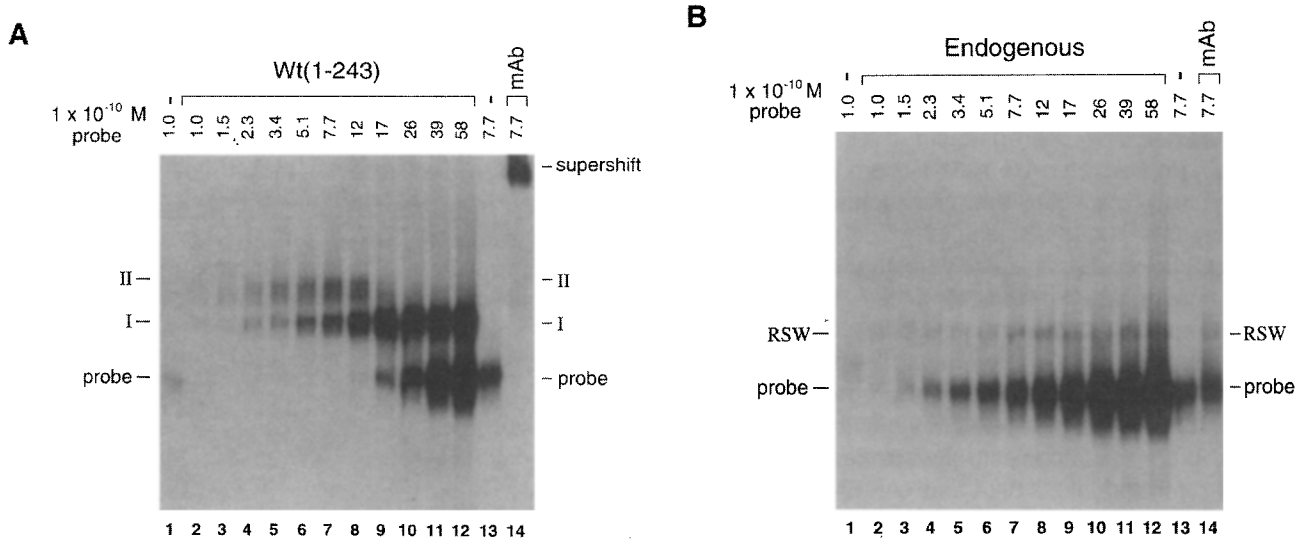


FIG. 2. Gel mobility-shift analysis of PKR Wt(1–243) as a function of $VA_I$ RNA probe concentration. (A) Recombinant PKR Wt(1–243). The Wt(1–243) protein concentration was kept constant at $7.0 \times 10^{-10}$ *M*, while the concentration of the $^{32}$P-labeled $VA_I$ RNA ligand was varied from $1 \times 10^{-10}$ to $5.8 \times 10^{-9}$ *M*. The I and II notation bordering the autoradiogram indicate the positions of the two complexes of shifted $VA_I$ RNA which were super-shifted by PKR monoclonal antibody (A, compare lanes 7 and 14). (B) Control protein preparation isolated from COS cells transfected with the pJC119 vector without insert. RSW indicates the position of the complex formed by an endogenous RNA-binding protein which was not super-shifted in the presence of monoclonal antibody to PKR (B, lane 14). Lanes 1 and 13 contain $^{32}$P-labeled $VA_I$ RNA probe with no added protein at a concentration of $1 \times 10^{-10}$ and $7.7 \times 10^{-10}$ *M*, respectively. Lanes 2–12 contain an increasing concentration of $^{32}$P-labeled $VA_I$ RNA with the indicated concentration in units of $1 \times 10^{-10}$ *M* above each respective lane. Lane 14, contains the same concentration of protein and $VA_I$ probe as lane 7, except monoclonal antibody to PKR was added after the protein–RNA complex was formed (A and B).

was present when the $VA_I$ RNA was in excess (Fig. 3A, lanes 7–9). By contrast, the RNA-binding activity of the K64E/K296R(1–551) protein was poor when compared to the activity of K296R(1–551) (Figs. 3B and 3A, respectively).

## Quantitation of Wt(1–243), K296R(1–551), and K64E/K296R(1–551) $VA_I$ RNA-binding data

The PKR dissociation constant of Wt(1–243) and K296R(1–551) for $VA_I$ RNA was determined by regression

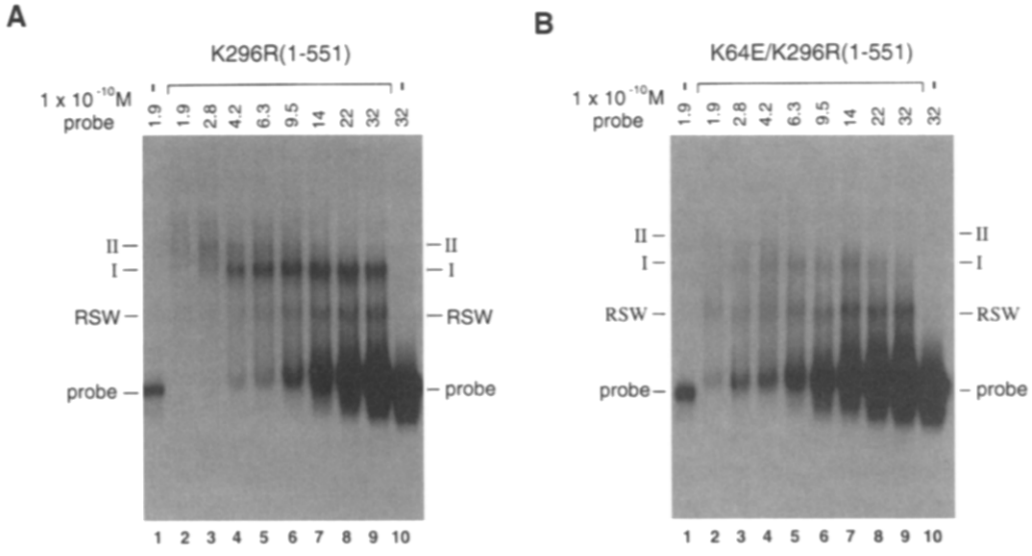


FIG. 3. Gel mobility-shift analysis of K296R(1–551) and K64E/K296R(1–551) as a function of $VA_I$ RNA probe concentration. The recombinant PKR proteins K296R(1–551) and K64E/K296R(1–551) were analyzed for their ability to bind $^{32}$P-labeled $VA_I$ RNA. The PKR protein concentration was held constant (lanes 2–9), while the concentration of $VA_I$ RNA ligand was varied from $1.9 \times 10^{-10}$ to $3.23 \times 10^{-9}$ *M*. (A) The K296R(1–551) protein was analyzed at the concentration of $4.0 \times 10^{-10}$ *M* (lanes 2–9). (B) The K64E/K296R(1–551) protein analyzed at the concentration of $3.0 \times 10^{-10}$ *M*. Lane 1 contains free $VA_I$ probe at a concentration of $1.9 \times 10^{-10}$ *M* with no added protein. Lanes 2–9 contain an increasing concentration of $^{32}$P-labeled $VA_I$ RNA with the indicated concentration in units of $1 \times 10^{-10}$ *M* above each respective lane. Lane 10 contains free probe at a concentration of $3.23 \times 10^{-9}$ *M* with no added protein. I and II, PKR specific complexes I and II; RSW, endogenous complex.

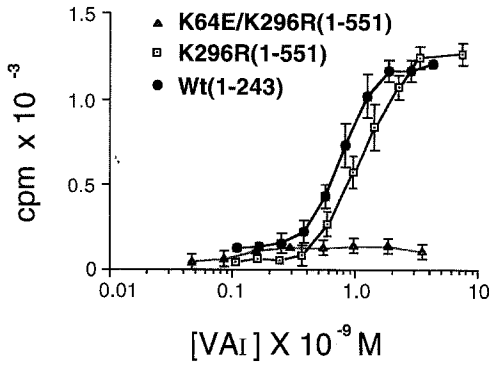


FIG. 4. Quantitation of PKR RNA-binding affinity to $VA_I$ RNA. Binding of $VA_I$ RNA to the Wt(1–243), K296R(1–551), and K64E/K296R(1–551) PKR proteins was performed as a function of $VA_I$ RNA concentration; quantitation was performed as described under Materials and Methods.

analysis of the relative amount of complex formed at varying concentrations of $^{32}$P-labeled $VA_I$ RNA (Fig. 4). The $K_D$ for $VA_I$ RNA calculated for the truncated Wt(1–243) PKR protein was 1.7 ± 0.6 × $10^{-9}$ *M*. The $K_D$ for full-length K296R(1–551) protein was comparable, 1.2 ± 0.3 × $10^{-9}$ *M*, as summarized in Table 1. The full-length $R_I$ motif mutant, K64E/K296R(1–551), bound $VA_I$ RNA very poorly (Fig. 4).

### RNA gel-shift competition analysis of Wt(1–243) and K296R(1–551) using pI:pC RNA competitor with $VA_I$ RNA probe

The dissociation constants of PKR for $VA_I$ RNA and pI:pC RNA were analyzed using a mobility-shift competition assay. The concentrations of both PKR protein and $VA_I$ RNA probe were held constant, and the concentration of a 200-bp pI:pC RNA competitor was varied (Fig. 5). The pI:pC effectively competed the binding of $^{32}$P-labeled $VA_I$ RNA, both to the truncated Wt(1–243) PKR protein (Fig. 5A) and the full-length K296R(1–551) PKR protein (Fig. 5B). The $K_D$ of Wt(1–243) for pI:pC RNA, measured by competition analysis, was 2.4 ± 0.1 × $10^{-9}$ *M;* the $K_D$ of K296R(1–551) for pI:pC RNA was 1.7 ± 1.0 × $10^{-9}$ *M* (Table 2).

### RNA gel-shift competition analysis of Wt(1–243) using both $VA_I$ and TAR RNA competitor with $VA_I$ RNA probe

The relative ability of adenovirus $VA_I$ RNA and HIV TAR RNA, added as $^{3}$H-labeled RNAs, to compete with the binding of $^{32}$P-labeled $VA_I$ RNA probe to PKR was examined. The binding of $^{32}$P-labeled $VA_I$ RNA to the Wt(1–243) (Fig. 6) and the K296R(1–551) (data not shown) recombinant proteins was much more effectively competed by $VA_I$ RNA than by TAR RNA. The $K_D$ of Wt(1–243) for $VA_I$ RNA by competition analysis was 1.6 ± 0.5 × $10^{-9}$ *M;* the $K_D$ of K296R(1–551) for $VA_I$ RNA by competition analysis was 3.6 ± 0.2 × $10^{-9}$ *M* (Table 2). The $K_D$ of Wt(1–243) and K296R(1–551) for TAR RNA were 1.6 ± 0.1 × $10^{-7}$ and 1.9 ± 0.2 × $10^{-7}$ *M*, respectively (Table 2).

## DISCUSSION

Three important points emerge from the results reported herein. The truncated Wt(1–243) and the full-length K296R(1–551) PKR proteins bound adenovirus $VA_I$ RNA with comparable efficiency as measured by a gel mobility-shift assay. However, the adenovirus $VA_I$ RNA was bound with approximately 100-fold greater affinity than was HIV TAR RNA by both Wt(1–243) and K296R(1–551) PKR proteins. Finally, the full-length K64E/K296R(1–551) PKR protein with an altered $R_I$ motif displayed a much lower relative binding activity for $VA_I$ RNA than did the full-length K296R(1–551) protein.

The quantitative nature of the results obtained by gel mobility-shift analysis of native PKR proteins extends our understanding of the RNA-binding activity of these forms of PKR deduced from Northwestern gel-blot analysis (McCormack *et al.*, 1994). The Wt(1–243) and K296R(1–551) proteins bound $VA_I$ RNA with comparable affinity, both proteins displaying a $K_D$ of approximately 1.4 × $10^{-9}$ *M* (Table 1). These results conclusively demonstrate that the necessary residues for efficient RNA-binding activity are contained within the N-terminal 243 amino acids of PKR. Likewise, the results suggest that the residues within the catalytic C-terminus of PKR do not significantly affect the RNA-binding properties attributed to the N-terminus of the protein.

Activation and/or inhibition of PKR autophosphorylation occurs upon RNA-binding (Katze, 1992; Mathews, 1993; Samuel, 1991). It is not definitively known whether the regulatory N-terminus of PKR acts as a steric or allosteric inhibitor of the catalytic C-terminus, but this appears to be a likely possibility. For example, the observation that ATP can be cross-linked to the PKR kinase only in the presence of dsRNA (Bischoff and Samuel, 1985; Galabru and Hovanessian, 1987) implies that the PKR protein undergoes a RNA-dependent conformational change which enables it to bind ATP. It also has been proposed that the $VA_I$ RNA functions as an inhibitor of PKR activation by eliciting a specific conformational

TABLE 1

DISSOCIATION CONSTANTS DETERMINED BY $VA_I$ RNA TITRATION ANALYSIS[a]

| PKR protein | RNA | $K_D$ |
|---|---|---|
| Wt (1–243) | $VA_I$ | 1.7 ± 0.6 × $10^{-9}$ *M* |
| K296R (1–551) | $VA_I$ | 1.2 ± 0.3 × $10^{-9}$ *M* |

[a] Constants derived from independent experiments similar to those shown in Figs. 2–4 as described under Materials and Methods.

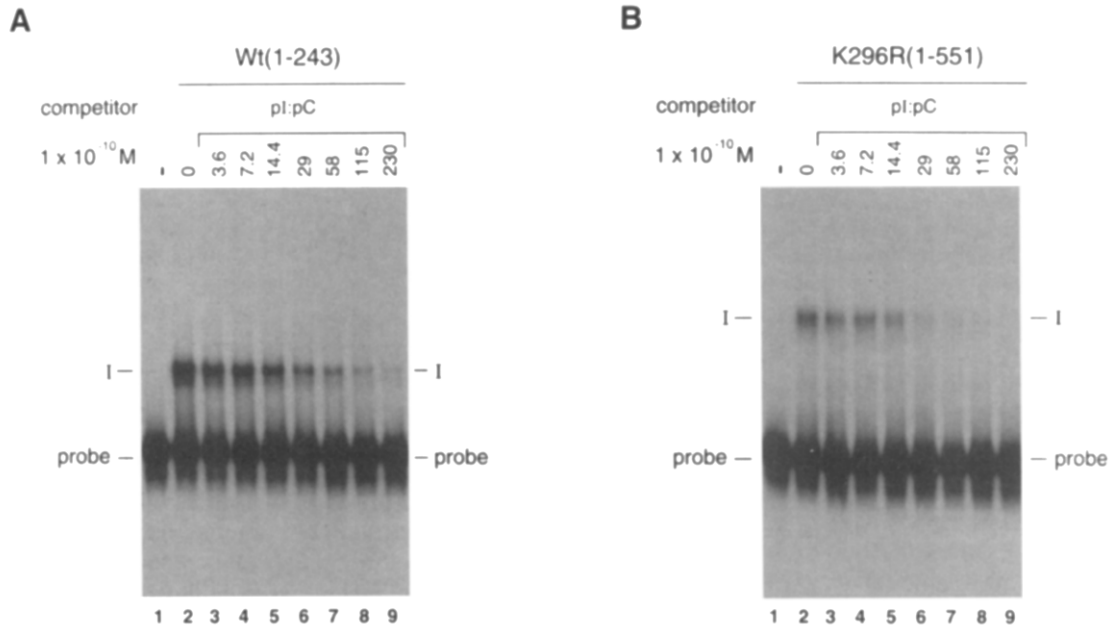


FIG. 5. Gel-shift competition analysis of PKR with $VA_I$ RNA as the probe and pI:pC RNA as the competitor. The concentrations of the recombinant PKR proteins and the $^{32}P$-labeled $VA_I$ RNA probe ($2.3 \times 10^{-9}$ *M*) were kept constant, and the amount of the pI:pC RNA competitor was varied. (A) Wt(1–243) protein at $5.8 \times 10^{-10}$ *M*. (B) K296R(1–551) protein at $2.4 \times 10^{-10}$ *M*. Lane 1, contains free probe; lane 2, no competitor added; lane 3, contains $3.6 \times 10^{-10}$ *M* pI:pC RNA competitor; lane 4, contains $7.2 \times 10^{-10}$ *M* pI:pC; lane 5, $1.4 \times 10^{-9}$ *M* pI:pC; lane 6, $2.8 \times 10^{-9}$ *M* pI:pC; lane 7, $5.8 \times 10^{-9}$ *M* pI:pC; lane 8, $1.15 \times 10^{-8}$ *M* pI:pC; and lane 9, $2.3 \times 10^{-8}$ *M* pI:pC RNA competitor.

change in the PKR protein, which thereby prevents autophosphorylation (Green and Mathews, 1992).

The RNA-binding activity displayed by K64E/K296R(1–551) PKR was substantially lower than that of K296R(1–551) PKR. We were unable to saturate the binding of $VA_I$ RNA to the K64E/K296R(1–551) double-mutant protein. It is not yet known if the K64E mutation significantly destabilizes the overall folding of PKR or alters the localized structure of the recombinant PKR protein, thereby resulting in impaired RNA-binding activity as measured both by gel mobility-shift analysis with native protein as described herein and by Northwestern analysis with renatured protein (McCormack *et al.,* 1994).

PKR appears to bind two distinct regions of $VA_I$ RNA, the double-stranded regions of the apical stem (Mellits *et al.,* 1990) and the central core of $VA_I$ RNA which has been implicated in the function of kinase inhibition (Furtado *et al.,* 1989; Ghadge *et al.,* 1991). The PKR protein contains two copies ($R_I$ and $R_{II}$) of the RNA-binding R motif. $R_I$ and $R_{II}$ seem to function as nonequivalent units; both copies are required for maximal RNA-binding activity (Green and Mathews, 1992; McCormack *et al.,* 1992, 1994). The motif $R_I$ is functional alone when expressed as a TrpE fusion protein, whereas the motif $R_{II}$ is not (McCormack *et al.,* 1992). Mutations in either motif $R_I$ or motif $R_{II}$ greatly reduce the RNA-binding activity of PKR (Green and Mathews, 1992; McCormack *et al.,* 1994). It is conceivable that the $\alpha$-helical motifs $R_I$ and $R_{II}$ each bind directly to distinct regions of $VA_I$ RNA and pI:pC RNA (Green and Mathews, 1992). However, the dissociation constants of Wt(1–243) and K296R(1–551) for pI:pC RNA and $VA_I$ RNA differed by less than twofold. This finding is consistent with the conclusion that the double-stranded regions of $VA_I$ RNA are indeed necessary for PKR binding (Mellits *et al.,* 1990). These results are also consistent with the notion that there is a single dsRNA binding site on PKR which is capable of binding both activator and inhibitor RNAs (McCormack *et al.,* 1992, 1994; Green and Mathews, 1992; Manche *et al.,* 1992).

Curiously, both the Wt(1–243) and K296R(1–551) proteins each complexed with $VA_I$ RNA to yield two unique complexes, designated I and II, detected by the gel-shift assay (Figs. 2A, 4A and 4B). This observation suggests dimerization of PKR may occur, mediated by the N-terminal region of the protein. The formation of complex II occurred only when the $VA_I$ RNA was limiting, either at

TABLE 2

DISSOCIATION CONSTANTS DETERMINED BY RNA COMPETITION ANALYSIS[a]

| PKR protein | RNA | $K_D$ |
|---|---|---|
| Wt (1–243) | $VA_I$ | $1.6 \pm 0.5 \times 10^{-9} M$ |
| K296R (1–551) | $VA_I$ | $3.6 \pm 0.2 \times 10^{-9} M$ |
| Wt (1–243) | TAR | $1.6 \pm 0.1 \times 10^{-7} M$ |
| K296R (1–551) | TAR | $1.9 \pm 0.2 \times 10^{-7} M$ |
| Wt (1–243) | pI:pC | $2.4 \pm 0.1 \times 10^{-9} M$ |
| K296R (1–551) | pI:pC | $1.7 \pm 1.0 \times 10^{-9} M$ |

[a] Constants derived from independent experiments similar to those shown in Figs. 5 and 6 as described under Materials and Methods.

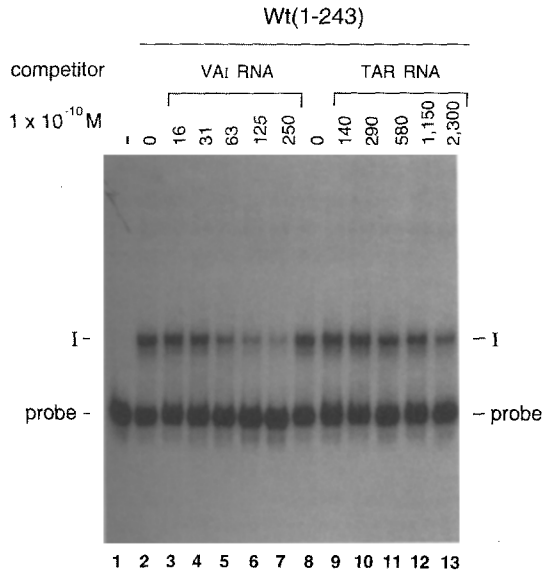


FIG. 6. Gel-shift competition analysis of PKR with $VA_I$ RNA as the probe and either $VA_I$ RNA or TAR RNA as the competitor. The concentration of PKR Wt(1–243) protein ($7.0 \times 10^{-10}$ *M*) and $^{32}$P-labeled $VA_I$ RNA probe ($2.7 \times 10^{-9}$ *M*) were kept constant, and the concentration of $^{3}$H-labeled RNA competitor was varied. Lane 1 contains free $VA_I$ RNA probe. Lanes 2 and 8 contain PKR protein and $VA_I$ RNA probe, with no TAR RNA competitor added. Lanes 3 to 7 contain increasing amounts of $^{3}$H-labeled $VA_I$ RNA competitor, and lanes 9 to 13 contain increasing amounts of $^{3}$H-labeled TAR RNA competitor.

high concentration of PKR protein (unpublished observations) or at low concentration of $VA_I$ RNA probe (Figs. 2A, 4A and 4B). The $VA_I$ RNA was shifted to a single complex under low concentrations of PKR protein [either K296R(1–551) or Wt(1–243)], and complex II was specifically formed when the $VA_I$ RNA probe was limiting. Both the bound I and II complexes are PKR specific, because they were both supershifted by monoclonal antibody to the PKR protein (Fig. 2A). By contrast, the endogenous RSW complex was not supershifted by the monoclonal antibody (Fig. 2B).

Manche *et al.* (1992) also observed multiple PKR–RNA complexes when examining dsRNA and partially purified PKR in a gel-shift assay; furthermore, the number of complexes increased with larger dsRNA probes. They proposed that the multiple-shifted complexes resulted from multiple PKR molecules binding to the same RNA molecule. Interestingly, we also detected both complex I and II when TAR RNA was used as the probe in the gel mobility-shift analysis instead of $VA_I$ RNA (unpublished results). Possibly the complex I represents monomeric PKR, whereas the complex II represents either a homodimer or a heterodimer of PKR. It is unlikely that the complex II represents nonspecific protein–protein oligomerization, because the complex was formed over a wide range of protein concentrations and was highly dependent on the concentration of the $VA_I$ RNA. Furthermore, nonspecific PKR RNA-binding would presumably be characterized by a much lower affinity than the specific binding of PKR to $VA_I$ RNA.

The dissociation constants for complexes I and II were comparable. However, when the stoichiometry of PKR protein to $VA_I$ RNA was 1:1, complex I was the predominant form (Figs. 2A and 4A). When the $VA_I$ concentration was increased, complex II disappeared while complex I increased in amount (Figs 2A and 3A). These results are consistent with PKR dimerization, which has been reported by Langland and Jacobs (1992). However, we cannot exclude the alternative possibility that the differences between complexes I and II arise from an alteration in PKR conformation, or post-translational modification of PKR, or by the association of PKR with other effector proteins. For example, the DNA-binding activity of the Fos and Jun proteins is altered by post-translational modification, by the nature of their dimerization, and by their association with specific effector proteins (Allegretto *et al.*, 1990).

Attempts were made to estimate the off-rate of the ligand $VA_I$ RNA, both with full-length PKR protein K296R(1–551) and the N-terminal RNA-binding domain Wt(1–243), using the gel-shift assay. When $^{32}$P-labeled $VA_I$ RNA–protein complexes were preformed before the addition of excess $^{3}$H-labeled $VA_I$ RNA, all of the $^{32}$P-labeled $VA_I$ RNA was displaced by the $^{3}$H-labeled $VA_I$ RNA within 1 min. However, an on-rate minimum of $5 \times 10^{6}$ mol/sec can be estimated for both the recombinant Wt(1–243) and K296R(1–551) proteins assuming that six half-lives had passed.

The explanation for the lower affinity of PKR for TAR RNA as compared to VA RNA is unclear. Perhaps the 82-nt single-stranded TAR RNA was either of insufficient size or present in incorrect context to form the optimal higher-ordered structure required for stable interaction with PKR. The size of dsRNA required to bind efficiently to PKR is estimated at approximately 85 bp, while duplexes of 40 bp bind only weakly (Manche *et al.*, 1992). Interestingly, the cellular TAR RNA-binding protein which contains the R motif binds TAR RNA with an affinity similar (Gatignol *et al.*, 1993) to that observed herein for PKR. Although shifted complexes I and II both contained PKR because they were both supershifted upon addition of PKR-specific monoclonal antibody, we cannot exclude the possibility that a second protein was also present in the complex in addition to PKR. The second protein might differentially affect the affinity of PKR for TAR and $VA_I$ RNAs.

In conclusion, we have shown that full-length [K296R(1–551)] and truncated [Wt(1–243)] PKR proteins were able to bind both $VA_I$ RNA and pI:pC RNA with comparable affinity, characterized by a $K_D$ of approximately $2.0 \times 10^{-9}$ *M* for both forms of PKR and both RNAs. By contrast, the binding of TAR RNA differed by about 100-fold, displaying a $K_D$ of approximately $2.0 \times 10^{-7}$ *M* with both forms of PKR. These results demon-

strate unequivocally that the different viral RNAs which affect PKR enzymic function may not necessarily interact with PKR in a quantitatively identical manner. Our understanding of the mechanism of PKR activation and inhibition will be facilitated by the identification of additional RNAs, including possibly cellular RNAs that modulate PKR activity, and by determination of the three-dimensional structure of PKR complexed with activator and inhibitor RNAs.

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